

Article

Optimization of an HRT-Fixed Plug-Flow Anaerobic-Oxic-Anoxic (AOA) Reactor: Changing the Specific Anaerobic/Oxic/Anoxic HRT Ratios

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Abstract: Anaerobic-Oxic-Anoxic (AOA) is a promising process that addresses the increasingly stringent requirements for advanced nitrogen (N) and phosphorus (P) removal in wastewater treatment plants (WWTPs). Plug-flow AOA systems have received much attention due to the similarity of their application scenarios to those of WWTPs; however, the understanding of the AOA process remains incomplete. In this study, a plug-flow AOA reactor was operated for 142 days under different A/O/A hydraulic retention time (HRT) ratios at a short HRT (13.3 h). Efficient nutrient removal performance was achieved at an A/O/A HRT ratio of 1:2:2, with total inorganic nitrogen (TIN), P, and chemical oxygen demand (COD) removal efficiencies of $78.3 \pm 5.5\%$, $96.0 \pm 3.7\%$, and $79.8 \pm 4.9\%$, respectively. Predominant functional bacteria, including *Candidatus_Competibacter* (2.1%) and *Defluviicoccus* (8.0%), as typical glycogen accumulating organisms (GAOs) contributed to good endogenous denitrification (approximately 37% TIN removal). Additionally, the reasonable A/O/A HRT ratio ensured synergistic interactions among multiple functional bacteria, enabling the stable operation of the efficient and cost-effective AOA system.

Keywords: Anaerobic-Oxic-Anoxic (AOA); plug-flow; hydraulic retention time (HRT); glycogen accumulating organisms (GAOs); endogenous denitrification (ED)



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1. Introduction

The increasingly stringent discharge standards present significant challenges for modern wastewater treatment plants (WWTPs) in achieving deep nitrogen (N) and phosphorus (P) removal [1–3]. Conventional biological nutrient removal (BNR) processes are increasingly unable to meet these strict requirements, particularly when taking into account energy/cost-savings under conditions of insufficient carbon sources [3]. Low carbon-to-N (C/N) ratios are a common issue in WWTPs, typically addressed by adding extra carbon sources—a solution that is often cost-ineffective [4]. To overcome these limitations, researchers have proposed various advanced, energy- and cost-efficient nutrient removal processes, among which the Anaerobic-Oxic-Anoxic (AOA) system has garnered significant attention [1,2,5,6]. The plug-flow AOA system, in particular, has attracted growing interest due to its compatibility with WWTPs application scenarios and its advanced utilization of endogenous carbon sources [1,7,8].

Compared to Anaerobic/Oxic (A/O) and Anaerobic-Anoxic-Oxic (AAO) processes, the AOA process demonstrates superior efficiency in reducing effluent total inorganic

nitrogen (TIN) through post-endogenous denitrification [2]. This improvement is primarily attributed to the activities of glycogen-accumulating organisms (GAOs) and phosphorus-accumulating organisms (PAOs). Both types of microorganisms transform organic matter into polyhydroxyalkanoates (PHAs) as intercellular carbon sources under anaerobic conditions, and utilize these intercellular carbon sources for endogenous denitrification (ED) under anoxic conditions [9]. Additionally, denitrifying P removal can also occur in the anoxic zone, facilitated by denitrifying PAOs (DPAOs) [2]. However, unlike PAOs, GAOs lack the ability to complete the P cycle, which involves releasing P in anaerobic conditions and subsequently taking it up in aerobic conditions. This limitation creates competition between two functional bacteria [9,10]. Furthermore, the AOA system integrates multiple functional groups, including ammonia oxide bacteria (AOB), nitrite oxide bacteria (NOB), denitrifying bacteria, PAOs, and GAOs, ensuring the efficient and stable removal of N and P [2,11].

To date, the adjustment of the AOA system has been investigated under various conditions, including low temperature [7], limited dissolved oxygen (DO) [12], and low C/N ratio [8]. Additionally, studies have explored the impacts of single or multiple sludge types [1] and coupling processes integrated with plug-flow AOA systems [1,11,13]. However, the optimization of the crucial parameter—hydraulic retention time (HRT)—has often been overlooked [14]. Liu et al. reported that extending anoxic HRT improved the N removal contribution of ED from 25% to 38%, and enhanced the TIN removal rate [1]. Conversely, an unreasonable A/O/A HRT ratio could result in impaired nitrification and disrupted cooperation among functional microbial groups [2]. Under such conditions, ammonia nitrogen ($\text{NH}_4^+\text{-N}$) cannot be fully oxidized, and the ED process may not be effectively performed. This imbalance could be further magnified when the HRT is limited. Wu et al. achieved HRT optimization and a high N removal rate of $63.2 \pm 9.3 \text{ g-N}/(\text{m}^3 \cdot \text{d})$ by gradually reducing HRT to a short duration of 5–6 h; however, this also led to an increase in effluent TIN [14]. Therefore, exploring and regulating the optional A/O/A HRT ratio is essential in maintaining an efficient and stable nutrient removal in plug-flow AOA systems. Furthermore, variations in microbial community structure should also be considered during the optimization process.

This study investigated the performance of a plug-flow AOA reactor by evaluating optional A/O/A HRT ratios under a fixed HRT of 13.3 h. The system's continuous operation was monitored over 142 days, focusing on the nutrient variations during long-operation and typical cycles across different phases. Additionally, high-throughput sequencing was employed to analyze changes in microbial community structure and the functional groups involved.

2. Materials and Methods

2.1. Experimental Equipment and Operation

The plug-flow system was operated in a polymethyl methacrylate reactor ($79 \times 17 \times 47 \text{ cm}$, working volume 40 L) (Figure 1). The reactor consisted of five equal compartments separated by fixed baffles (each with a permeable hole), and each compartment was equipped with a mechanical stirrer. A sedimentation tank with a working volume of 19 L was used for sludge settling and recirculation. During the entire operation, the influent flow rate was maintained at 3 L/h, resulting in a total HRT of 13.3 h. The sludge reflux ratio was set to 50%, and the sludge retention time (SRT) was maintained at 100 days by regularly discharging 400 mL/d of the aerobic zone mixed liquor. The aeration capacity was controlled using a gas flow meter and maintained at 0.8 L/min. The temperature was uncontrolled and remained at room temperature ($21.5 \pm 2.5 \text{ }^\circ\text{C}$), while the pH was approximately 7.5 without regulation.

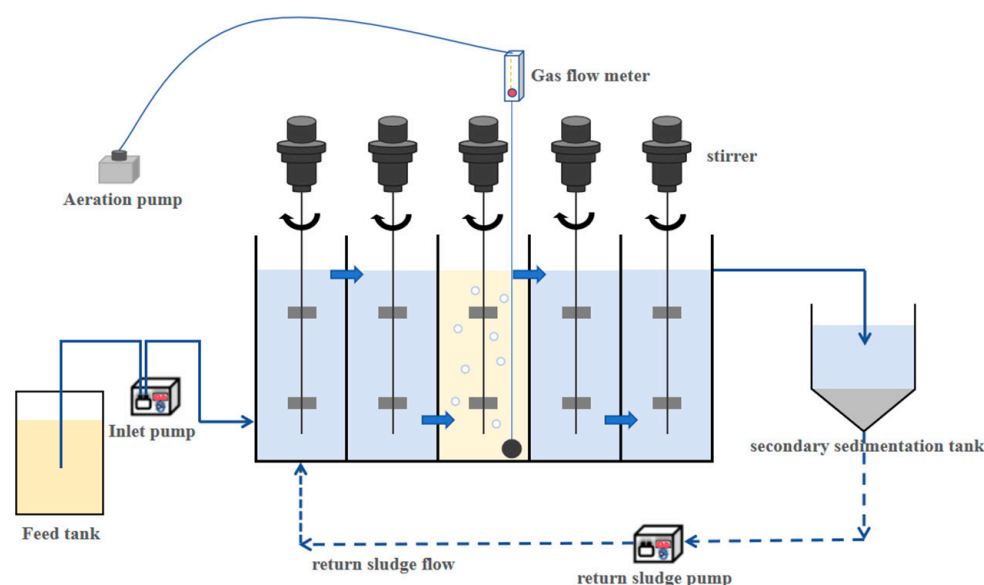


Figure 1. Experimental device of plug-flow system (Phase 1).

The plug-flow reactor was continuously operated for 142 days under AOA mode, divided into six phases (Table 1). In Phase 1 (days 1–27), the Anaerobic/Oxic/Anoxic HRT (A/O/A HRT) ratio was set at 2:1:2. This was adjusted to 1:2:2 for days 28–63 (Phase 2). This A/O/A HRT ratio (volume ratio) remained unchanged from Phase 2 to Phase 4, while the influent $\text{NH}_4^+\text{-N}$ concentration was kept constant. In contrast, the COD concentration was adjusted to optimize nutrient removal performance, leading to an influent COD/ $\text{NH}_4^+\text{-N}$ (C/N) ranging from 3.6 ± 0.4 to 4.8 ± 0.3 . In Phase 5 (days 116–125), the A/O/A HRT ratio was modified to 1:1:3 to strengthen ED capacity. Subsequently, it reverted to 1:2:2 in Phase 6 due to the dramatically deteriorated nutrient removal performance (days 125–142).

Table 1. Operational conditions for the plug-flow system.

Item	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6
	1–27 d	28–63 d	64–81 d	82–116 d	116–125 d	125–142 d
Operational mode	AOA	AOA	AOA	AOA	AOA	AOA
Volume ratio	2:1:2	1:2:2	1:2:2	1:2:2	1:1:3	1:2:2
Anaerobic HRT (h)	5.32	2.66	2.66	2.66	2.66	2.66
Oxic HRT (h)	2.66	5.32	5.32	5.32	2.66	5.32
Anoxic HRT (h)	5.32	5.32	5.32	5.32	7.98	5.32
Influent COD (mg/L)	221 ± 13	210 ± 5	285 ± 19	250 ± 23	265 ± 17	246 ± 11
Influent C/N	3.9 ± 0.2	3.6 ± 0.4	4.8 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.0 ± 0.2
Influent C/P	44.3 ± 8.6	40.3 ± 6.3	56.9 ± 8.4	49.7 ± 4.0	55.1 ± 5.7	49.6 ± 3.8

2.2. Wastewater and Inoculated Sludge

This study used synthetic wastewater prepared from tap water and other components. The composition of wastewater used for the plug-flow system was as follows: $\text{NH}_4^+\text{-N}$ (provided by NH_4Cl), 59.0 ± 2.6 mg/L; $\text{PO}_4^{3-}\text{-P}$ (provided by KH_2PO_4), 5.1 ± 0.6 mg/L; chemical oxygen demand (COD, provided by NaAc), 210 ± 5 – 285 ± 19 mg/L; NaHCO_3 , 300 mg/L; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 14 mg/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 90 mg/L; trace element solution, 2 mL/L (Table S1).

The inoculated sludge was excess sludge with good N and P removal capabilities, obtained from a lab-scale plug-flow AOA reactor [2] and stored at 4 °C before the experiment.

2.3. Chemical Analysis Method

All collected mixed liquor samples were filtered through 0.45 μm filter paper prior to analysis. $\text{NH}_4^+\text{-N}$, nitrite–nitrogen ($\text{NO}_2^-\text{-N}$), and nitrate–nitrogen ($\text{NO}_3^-\text{-N}$) were

analyzed using an ultraviolet spectrophotometer (UV-5200, Shanghai, China) following the standard methods [15]. TIN was calculated from the sum of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$. Orthophosphorus ($\text{PO}_4^{3-}\text{-P}$) was also analyzed using standard methods [15]. COD was determined by use of a COD rapid analyzer (Lianhua Technology Co., Ltd., 5B-3A, Shanghai, China). Both temperature and pH were measured using a pH meter (pH/AZ8602, Taiwan, China).

2.4. Calculations

Simultaneous nitrification and denitrification (SND) efficiency is defined as the nitrogen loss in the aerobic compartment, calculated using Equation (1) [9].

$$\text{SND efficiency} = \left(1 - \frac{\Delta\text{NO}_2^- + \Delta\text{NO}_3^-}{\Delta\text{NH}_4^+} \right) \times 100\% \quad (1)$$

where ΔNO_2^- and ΔNO_3^- are the production of $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ in the aerobic compartment(s), respectively (mg/L); ΔNH_4^+ is the total $\text{NH}_4^+\text{-N}$ consumption in the aerobic compartment(s), mg/L.

$\text{COD}_{\text{intra}}$ is defined as the intracellular carbon source (stored by COD) in the anaerobic compartment(s) of endogenous partial denitrification, excluding heterotrophic denitrification consumption (namely, COD_{dn}) (calculated by Equations (2) and (3)) [9]. Additionally, $\text{COD}_{\text{intra}}$ efficiency is calculated using Equation (4), which is defined as the proportion of $\text{COD}_{\text{intra}}$ in total anaerobic COD consumption ($\Delta\text{COD}_{\text{An}}$).

$$\Delta\text{COD}_{\text{An}} = \text{COD}_{\text{i,An}} - \text{COD}_{\text{e,An}} = \text{COD}_{\text{dn}} + \text{COD}_{\text{intra}} \quad (2)$$

$$\text{COD}_{\text{dn}} = 1.71\Delta\text{NO}_2^-\text{-N}_{\text{An}} + 2.86\Delta\text{NO}_3^-\text{-N}_{\text{An}} \quad (3)$$

$$\text{COD}_{\text{intra}} \text{ efficiency}(\%) = (\text{COD}_{\text{intra}} / \Delta\text{COD}_{\text{An}}) \times 100\% \quad (4)$$

where $\text{COD}_{\text{i,An}}$ and $\text{COD}_{\text{e,An}}$ are the theoretical initial COD concentrations and COD concentration in the anaerobic compartment(s), respectively, mg/L; 1.71 and 2.86 are the theoretical values of COD consumption for denitrifying per unit $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$, respectively, mgCOD/mgN; $\Delta\text{NO}_2^-\text{-N}_{\text{An}}$ and $\Delta\text{NO}_3^-\text{-N}_{\text{An}}$ are the theoretical decreases in $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ in the anaerobic compartment(s), respectively, mg/L.

2.5. Illumina MiSeq Sequencing

Illumina high-throughput sequencing was used to analyze microbial community changes in nine sludge samples collected on days 1, 27, 63, 81, 116, 125, and 142, named Day_1, Day_27, Day_63, Day_81, Day_116, Day_125, and Day_142, respectively. All the sludge samples were collected from the last anoxic compartment (AN2 or AN3). DNA extraction was performed using the Fast DNA SPIN Kit (MP Biomedicals, Santa Ana, CA, USA). The V3-V4 region of the bacterial 16S rRNA gene was amplified with the 338F/806R primers. The purified amplicons were sequenced on the Illumina MiSeq PE300 platform (Illumina, Santa Ana, CA, USA) at Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China).

3. Results and Discussion

3.1. Comparing the Nutrient Removal Under Different A/O/A HRT Ratio

In Phase 1, the A/O/A HRT ratio was set at 2:1:2 according to the standards of Xu et al. [2], with the expectation of achieving a rapid startup of the AOA system in the plug-flow reactor. The average influent COD concentration was 220.6 mg/L, resulting in a C/N close to 3.9 ± 0.2 . The effluent COD concentration (Eff. COD) was approximately

42.5 ± 7.4 mg/L, achieving a removal efficiency of $80.7 \pm 3.2\%$ (Figure 2A). The effluent concentration of $\text{PO}_4^{3-}\text{-P}$ (Eff. $\text{PO}_4^{3-}\text{-P}$) was below 2.15 mg/L, except slight fluctuations observed on days 13 and 17, leading to a good P removal ($87.9 \pm 11.2\%$) (Figure 2B). This performance was attributed to the abundant presence of PAOs in the seed sludge, which was inoculated from domesticated lab-sludge. However, the effluent $\text{NH}_4^+\text{-N}$ (Eff. $\text{NH}_4^+\text{-N}$) started at 31.6 mg/L on day 1 and peaked at 41.9 mg/L on day 17, indicating unsatisfactory $\text{NH}_4^+\text{-N}$ removal during Phase 1 (approximately 60%). Meanwhile, the TIN removal efficiency increased from 43.6% to 76.4% during this period (Figure 2D), but it was primarily limited by poor $\text{NH}_4^+\text{-N}$ removal rather than $\text{NO}_3^-\text{-N}$, as the effluent $\text{NO}_3^-\text{-N}$ (Eff. $\text{NO}_3^-\text{-N}$) decreased from 5.56 mg/L on day 1 to below 1.12 mg/L by day 15. Li et al. reported that gradually reducing the aerobic HRT would deteriorate the $\text{NH}_4^+\text{-N}$ removal performance, achieving ~50% removal efficiency in an AOA mode sequencing batch reactor [12]. Hence, extending the aerobic HRT by reducing the anaerobic HRT could be a promising method, as COD was almost entirely consumed in the first anaerobic compartment (AN1), and P release peaked (Figure 3A), indicating redundancy in the anaerobic compartments.

Therefore, the A/O/A HRT ratio was adjusted to 1:2:2 in Phase 2, resulting in a change in non-aerated HRT/aerated HRT from 4 to 1.5 to improve $\text{NH}_4^+\text{-N}$ removal efficiency [7]. After a brief adaption (within 3 days), the Eff. $\text{NH}_4^+\text{-N}$ dropped below 0.9 mg/L, achieving an $\text{NH}_4^+\text{-N}$ removal efficiency of over 99% (Figure 2C), which is comparable with that of previous AOA systems [2,14,16]. During this phase, despite the reduction in one anaerobic compartment and a shorter anaerobic HRT (Table 1), the Eff. COD remained largely unchanged at 36.8 ± 6.3 mg/L, with a removal efficiency of $82.2 \pm 2.8\%$. This finding is consistent with that of Zhao et al. [5], indicating an abundance of heterotrophic microorganisms in the AOA system. Additionally, the $\text{COD}_{\text{intra}}$ efficiency increased slightly from $91.1 \pm 2.0\%$ in Phase 1 to $95.9 \pm 2.8\%$ in Phase 2 (Figure 2A), indicating that shortening anaerobic HRT had a minimal effect on the storage of intracellular carbon sources [5]. However, the $\text{PO}_4^{3-}\text{-P}$ removal efficiency ($94.4 \pm 0.1\%$) remained stable before day 54 (Figure 2B), and then deteriorated with an increase in Eff. $\text{PO}_4^{3-}\text{-P}$. This was attributed to the increased activity of NOB and unstable COD influent, which caused a greater $\text{NO}_3^-\text{-N}$ concentration to accumulate in the initial anaerobic compartment for denitrification. Therefore, the storage of $\text{COD}_{\text{intra}}$ was lower than before, which contributed to an upward trend in Eff. $\text{PO}_4^{3-}\text{-P}$ from days 54 to 61. Interestingly, Eff. $\text{NO}_3^-\text{-N}$ rapidly rose to 28.4 mg/L on day 43 after the aerobic HRT was increased, which was attributed to the increased nitrifiers (AOB and NOB) activity. Consequently, Eff. TIN showed a noticeable increase during this period, despite the high $\text{NH}_4^+\text{-N}$ removal efficiency. From day 44, fluctuations in the oxygen supply occurred due to high voltage load in the laboratory, which led to the formation of more anoxic zones in the aerobic compartment for SND, contributing to temporary abnormal TIN concentration (day 50–57), while Eff. $\text{NO}_3^-\text{-N}$ (~21 mg/L), which was close to the oxic end $\text{NO}_3^-\text{-N}$ concentration, indicated a weak ED capacity. This might be because the $\text{COD}_{\text{intra}}$ was depleted after passing through the aerobic compartments.

To further optimize the AOA system, the influent COD concentration was increased by 100 mg/L in Phase 3, achieving a C/N of 4.8 ± 0.3 . Despite the adjustment, the COD removal efficiency remained stable at $85.3 \pm 3.0\%$ (Figure 2A), indicating a robust COD removal capacity in the AOA system [17]. The $\text{NH}_4^+\text{-N}$ removal efficiency showed a similar pattern (Figure 2C), whereas Eff. $\text{PO}_4^{3-}\text{-P}$ sharply increased to 6.33 mg/L on day 73, resulting in a reduced P removal ($34.6 \pm 13.9\%$) (Figure 2B). As reported by Chen et al., external COD could deteriorate P removal and polyhydroxyalkanoates consumption, leading to a vicious cycle for P removal ability [18]. However, TIN removal efficiency

increased gradually from 64.3% to 77.8% (Figure 2D), as Eff. NO_3^- -N declined from 20.4 mg/L to 12.4 mg/L over 18 days, showing obvious differences compared to oxic end NO_3^- -N concentration (~ 7.5 mg/L) (Figure 2E). This implies the ED process was mainly facilitated by GAOs, which outcompeted PAOs for intracellular carbon sources. As reported by Song et al., an increased COD/phosphorus (C/P) ratio (from 25 to 40) could lead to GAOs occupying dominant position and outweighing the PAOs [19]. In this study, the C/P ratio was increased from 40.3 ± 6.3 to 56.9 ± 8.4 , intensifying the competition for carbon sources between PAOs and GAOs and ultimately deteriorating PO_4^{3-} -P removal efficiency [5]. Additionally, the decrease in NO_3^- -N concentration in anoxic compartments further confirmed that GAOs might utilize more intracellular carbon source, as $\text{COD}_{\text{intra}}$ efficiency remained steady at $98.0 \pm 2.0\%$ (Figure 2A). This phenomenon is further explained through microbial community analysis (discussed in Section 3.3). In Phase 4, the A/O/A HRT ratio was kept at 1:2:2 (Table 1), while the influent COD concentration was reduced to 250 ± 23 mg/L, resulting in a C/N of 4.3 ± 0.3 . Although $\text{COD}_{\text{intra}}$ and Eff. NH_4^+ -N remained stable at $98.5 \pm 1.2\%$ and 1.9 ± 3.5 mg/L, respectively, the PO_4^{3-} -P removal efficiency did not return to its previous level, with Eff. PO_4^{3-} -P showing a decreased trend, reaching less than 2.8 mg/L by the end of this phase (Figure 2). This suggests that GAOs, which held an advantaged position in the competition for carbon sources, adversely impacted P removal despite operational parameters having been changed. This observation was further evidenced by the anoxic NO_3^- -N removal, which was mainly attributed to endogenous denitrification (Figure 2E). However, Song et al. suggested that an EBPR system with dominant GAOs could also achieve an efficient P removal of 75.9% under anaerobic/aerobic mode [19]. Additionally, excellent nitrification enhanced the endogenous denitrification process [2,7], contributing to a high TIN removal efficiency of $74.3 \pm 6.3\%$ (Figure 2D).

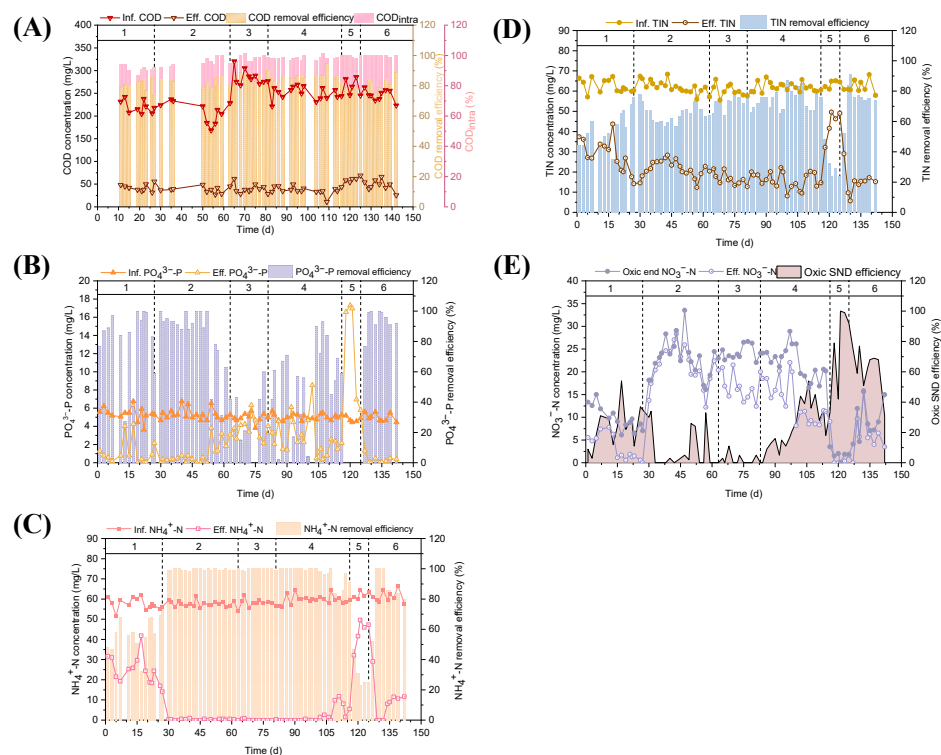


Figure 2. The long-term nutrient removal performances of COD (A), PO_4^{3-} -P (B), NH_4^+ -N (C), TIN (D) and NO_3^- -N (E).

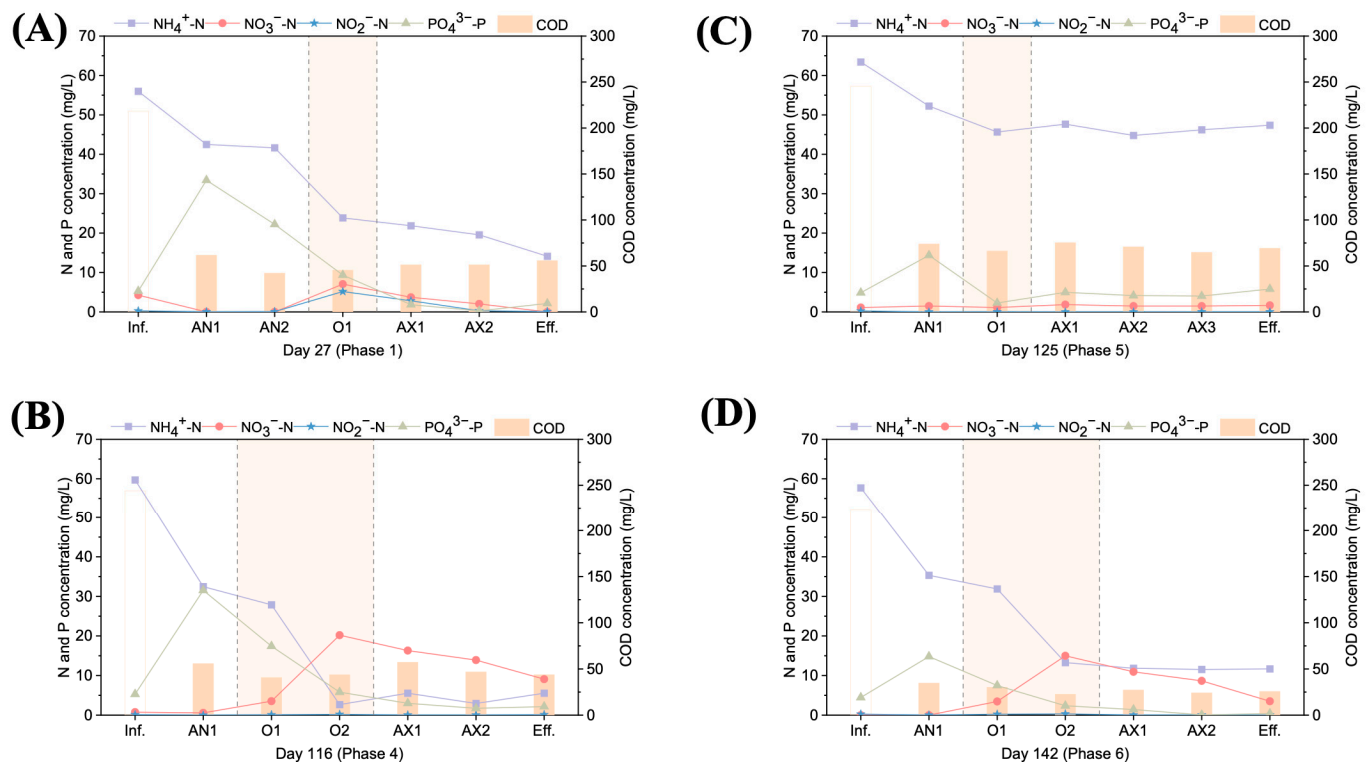


Figure 3. The variations of nutrients on day 27 (A), day 116 (B), day 125 (C), and day 142 (D).

Xu et al. suggested that a prolonged aerated HRT can lead to complete nitrification [2], generating a large amount of NO_3^- -N, which can accumulate in the initial anaerobic compartment via internal recycling (Figure 1). This accumulated NO_3^- -N increases carbon consumption for denitrification, thereby reducing the carbon available for anaerobic P release in the anaerobic compartment. Moreover, excessive aeration results in increased energy consumption. To balance these, in Phase 5, the aerated HRT was reduced to 2.66 h (Table 1) to save energy. However, despite achieving an SND efficiency of ~80%, Eff. NH_4^+ -N rapidly increased up to 49.7 on day 121 mg/L (Figure 2C). The increased anoxic HRT (Table 1) failed to further improve the TIN removal efficiency, resulting in significantly inadequate N and P removal (Figure 2). This was mainly attributed to the limited aerated HRT [17]. Consequently, the non-aerated/aerated HRT rapidly reverted to 1.5 (A/O/A HRT ratio of 1:2:2) after 9 days (Phase 6). While the COD removal efficiency was unaffected ($79.8 \pm 4.9\%$), PO_4^{3-} -P and NH_4^+ -N removal efficiencies recovered to $96.0 \pm 3.7\%$ and $89.6 \pm 8.3\%$, respectively, with Eff. PO_4^{3-} -P and Eff. NH_4^+ -N values of 0.2 ± 0.2 mg/L and 6.4 ± 5.1 mg/L, respectively (Figure 2). These results are inconsistent with those of Xu et al. [2], possibly because the temporary lack of dissolved oxygen (for only 9 days) had a minimal impact on the AOA system's operation. Additionally, the Eff. NO_3^- -N decreased to 3.5 mg/L within 13 days with good SND efficiency (~60%), contributing to a higher TIN removal efficiency ($78.3 \pm 5.5\%$) (Figure 2D,E). This confirms that the AOA system had a certain degree of recovery capacity in nutrient removal, supporting the findings of Gao et al. [7].

Overall, the long-term operation of the plug flow AOA system demonstrated that an A/O/A HRT ratio of 1:2:2 was critical in achieving stable and efficient N and P removal. Furthermore, the system consistently exhibited robust COD removal capacity, suggesting that the A/O/A HRT ratio had a minimal impact on COD removal in the plug-flow AOA system.

3.2. Nutrients Removal Mechanisms in Various Non-Aerated/Aerated HRT

The N and P removal mechanisms were further analyzed by typical cycles of the nutrient concentrations in different compartments at the end of selected phases (Phase 1, 4, 5, and 6). On day 27 (Phase 1, Figure 3A), under an A/O/A HRT ratio of 2:1:2, the COD concentration significantly decreased to 61.7 mg/L in the AN1 compartment, and showed a slight decline in the AN2 compartment. Furthermore, PO_4^{3-} -P concentration peaked at 33.4 mg/L in the AN1 compartment instead of AN2, suggesting that the additional anaerobic compartment (AN2) was unnecessary. Interestingly, the PO_4^{3-} -P concentration showed a slight decrease in the AN2 compartment, which could be attributed to the existence of microalgae releasing oxygen [20] or back-mixing phenomena between the adjacent compartments [2]. However, the NH_4^+ -N concentration remained stable in both the AN1 and AN2 compartments (approximately 42 mg/L), and decreased to 23.9 mg/L in the O1 compartment due to insufficient oxygen supply. Nevertheless, a slight decrease in NH_4^+ -N concentration was also observed from the AX1 compartment to the sedimentation tank. This phenomenon was also observed in other plug-flow AOA systems [7], which might be attributed to a small amount of dissolved oxygen mixing into the subsequent compartments with the water flow. Additionally, a certain amount of NO_2^- -N and NO_3^- -N accumulated in the O1 compartment (sum of 12.15 mg/L), alongside the oxidization of NH_4^+ -N (17.8 mg/L), indicating the occurrence of SND (Figure 2E). Short aerobic HRT (2.66 h) and low aeration (0.8 L/min) contributed to the good SND, and were blamed for the poor NH_4^+ -N removal. Moreover, the reduction in NO_2^- -N, NO_3^- -N, and PO_4^{3-} -P in the anoxic compartments suggested the feasibility of anoxic P uptake and endogenous denitrification in the plug-flow AOA system (Figure 4A).

In Phase 4 (day 116), one anaerobic compartment was removed and replaced with an aerobic compartment, achieving an aerobic HRT of 5.32 h (Table 1). The COD was effectively removed in the AN1 compartment despite the reduced anaerobic HRT, supporting the opinion of Zhao et al. [5]. PO_4^{3-} -P and NH_4^+ -N showed decreases in aerobic compartments (O1 and O2), although their decreases were less pronounced in the O1 compartment (Figure 3B). This indicates that the aerated time was sufficient to nitrify bacterium and PAOs. Correspondingly, the NO_3^- -N concentration increased to 20.2 mg/L in the O2 compartment and gradually decreased in the anoxic compartments and sedimentation tank (to approximately 9.1 mg/L), indicating effective endogenous denitrification driven by denitrifying GAOs (DGAOs). Additionally, PO_4^{3-} -P concentration only decreased by 3.4 mg/L in anoxic compartments, so the ED process contributed to approximately 37% TIN removal. However, when the aerobic HRT was reduced and the anoxic HRT was increased (Phase 5, day 125) to enhance anoxic P uptake and ED, the nutrient removal performance deteriorated significantly in the AOA system, except for COD (Figure 2). Only slight nitrification occurred (Figure 3C), oxidating 4.6 mg/L NH_4^+ -N, with no detectable generation of NO_2^- -N or even NO_3^- -N, which was attributed to the SND capacity (Figure 2E). The amount of P released decreased to 9.5 mg/L in the AN1 compartment, compared to 28.3 mg/L in Phase 1 and 26.2 mg/L in Phase 4, but was similar to that in Phase 6, suggesting that dominant GAOs might outcompete PAOs in the carbon competition. The PO_4^{3-} -P removal efficiency was also poor—as low as 0 (Figure 3C). Therefore, sufficient aerobic HRT needs to be ensured, as it plays a crucial role in maintaining stable long-term AOA operation [2].

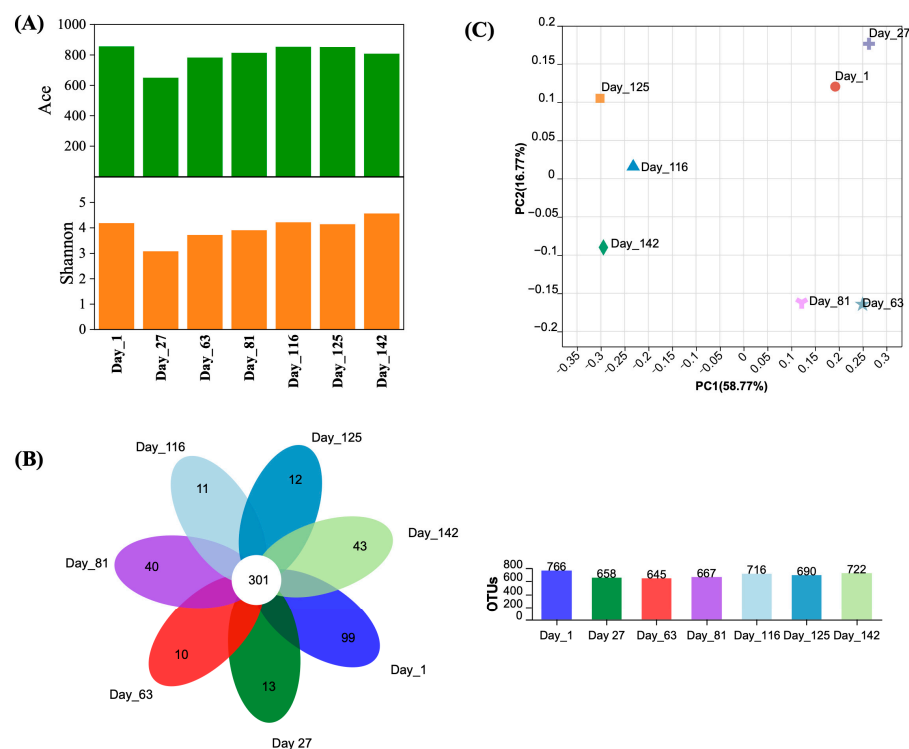


Figure 4. The alpha diversity indexes (A). OT numbers shared among seven bio-samples with the OTUs numbers in different bio-samples (B). Principal Coordinate Analysis (PCoA) with Bray–Curits distance among seven bio-samples (C).

On day 142 (Phase 6), the aerobic HRT was adjusted back to its previous level (5.33 h). Nevertheless, incomplete $\text{NH}_4^+\text{-N}$ oxidation in the O2 compartment resulted in approximately 11 mg/L $\text{NH}_4^+\text{-N}$ remaining in subsequent compartments (Figure 3D), indicating a slight decrease in nitrification performance. As reported by Xu et al., nitrification could be weakened by the combined effects of low dissolved oxygen and prolonged unaerated duration [2]. Thus, carefully setting a reasonable A/O/A HRT ratio is crucial to prevent irretrievable deterioration in nitrification. Fortunately, the $\text{PO}_4^{3-}\text{-P}$ uptake capacity remained stable, achieving complete P removal in the sedimentation compartment, with 2.3 mg/L $\text{PO}_4^{3-}\text{-P}$ taken up by denitrification P removal (DPR). Additionally, the $\text{NO}_3^-\text{-N}$ concentration decreased by 6.3 mg/L from the O2 compartment to the AX2 compartment, even though COD was nearly depleted in the AN1 compartment, contributing to good TIN removal efficiency and partially compensating for the deteriorated nitrification. This might be attributed to stable uptake of P by PAOs and ED by DGAOs (discussed in Section 3.3).

In summary, a reasonable A/O/A HRT ratio was necessary for the stable operation of the plug-flow AOA system. It was conducive to the synergism of functional bacterium in the AOA system, facilitating efficient N and P removal, as well as satisfactory ED process.

3.3. Microbial Community Profile

3.3.1. Microbial Community Diversity

During the 142-day operation of the plug-flow AOA system, seven bio-samples were collected and labeled as Day_1, Day_27, Day_63, Day_81, Day_116, Day_125, and Day_142. A total of 38,465–63,881 sequences were obtained from these bio-samples, clustered into 1212 OTUs (Table S2), and classified into 35 phyla and 460 genera. Additionally, the coverage exceeded 0.996 (Table S2), indicating high accuracy and reliability [2].

Alpha diversity was assessed using the Ace and Shannon indexes (Figure 4A). In Phase 1 (Day_1 to Day_27), both the Ace and Shannon indexes showed a marked decrease,

suggesting that richness and diversity were slightly affected. This could be attributed to a certain proportion of bacteria initially struggling to adapt to the laboratory environment, as also reported by Nie et al. [21]. Additionally, Xu et al. reported that limited aerated HRT could lead to the loss of aerobic microorganisms [2], attributed to the unreasonable A/O/A HRT ratio in this study. After adjusting the non-aerated/aerated HRT ratio to 1.5 (A/O/A HRT ratio of 1:2:2), richness and diversity increased in Day_63 (Phase 2). Furthermore, the Ace and Shannon indexes showed a slight increase in Day_81 (Phase 3), attributed to the increased influent C/N (Table 1), which enriched more heterotrophic organisms. Subsequently, a brief non-aerated/aerated HRT ratio of 4 (A/O/A HRT ratio of 1:1:3) was implemented (Table 1), resulting in deteriorated N and P removal performance; however, the Ace and Shannon indexes remained relatively stable in the subsequent phases. This suggests that the AOA system had a certain degree of robust recovery capacity under temporary adverse conditions. Moreover, a reasonable A/O/A HRT ratio could maintain richness and diversity within the community.

The individual bio-samples contained 645–766 OTUs (Figure 4B), with 301 OTUs shared among all the bio-samples. Additionally, the unique OTUs for the seven bio-samples were 99, 13, 10, 40, 11, 12, and 43, respectively. The Principal Coordinate Analysis (PCoA) further illustrated the variations in the microbial community. As shown in Figure 4C, 75.54% of the variance was explained by PC1 (58.77%) and PC2 (16.77%). Day_63 was distant from the bio-samples in Phase 1 (Day_1 and Day_27), indicating significant changes in the microbial community structure after adjusting the A/O/A HRT ratio (Table 1). However, Day_81 was only moderately distant from Day 63 (Figure 4C), despite the increase in influent C/N ratio from 3.6 ± 0.4 to 4.8 ± 0.3 (Table 1). This may be attributed to the fact that the regulated strategy was implemented over a relatively short period (18 days), while Day_116 was distant from Day_81 due to its nearly doubled phase duration (35 days). Additionally, Day_125 was significantly different from Day_142 (Figure 4C), compared to Day_116, suggesting that insufficient aerobic HRT might have affected the microbial community structure even over a relatively short period (9 days).

3.3.2. Microbial Population Dynamics

As shown in Figure 5A, Proteobacteria (22.3–74.6%), Bacteroidota (5.5–22.6%), and Chloroflexi (4.9–36.8%) were the consistently dominant phyla (>5%) throughout the study period, consistent with previous AOA-related systems [2,5,7,11]. In Day_1, the microbial community was mainly composed of Proteobacteria (61.2%), Bacteroidota (13.8%), Chloroflexi (9.4%), Patescibacteria (3.7%), and Firmicutes (3.5%). Among these, the relative abundance of Proteobacteria slightly increased in Phase 1 (Day_27, 74.6%), and then decreased gradually to 22.3% by Day_142, mainly due to declines in *unclassified_f_Beijerinckiaceae* (from 46.3% to 3.0%) and *Candidatus_Competibacter* (from 11.1% to 2.1%). Moreover, the relative abundance of Bacteroidota showed an overall increasing trend, despite a noticeable decrease in Day_63 (mainly contributed by *norank_f_Saprospiraceae*, *norank_f_SB-5*, and *Terrimonas*), which could be attributed to the operational mode change (A/O/A HRT ratio from 2:1:2 to 1:2:2). Similarly, the relative abundance of Chloroflexi increased to 36.8% in Day_142, becoming the most dominant phylum. Interestingly, slight declines were observed in Day_27 and Day_125, primarily attributed to decreases in *OLB13* and *unclassified_c_Anaerolineae*. In previous studies [22,23], *OLB13* was commonly observed in a partial denitrification system and possesses a denitrifying capacity. Hence, a plausible reason for the decrease in Chloroflexi is that insufficient aerated HRT could weaken nitrification and NO_3^- -N generation, negatively affecting the subsequent anoxic denitrification process. Additionally, Firmicutes exhibited a dramatic rise in Day_125 (15.9%), primarily due to an increase in *Trichococcus* (up to 15.8%). The other phyla, including Acidobacteriota (1.3–3.3%),

Actinobacteriota (1.0–3.6%), Planctomycetota (0.6–1.7%) and Latescibacterota (0.4–1.3%), were also observed in previous AOA-related systems [2], but exhibited relatively minor variations in relative abundance.

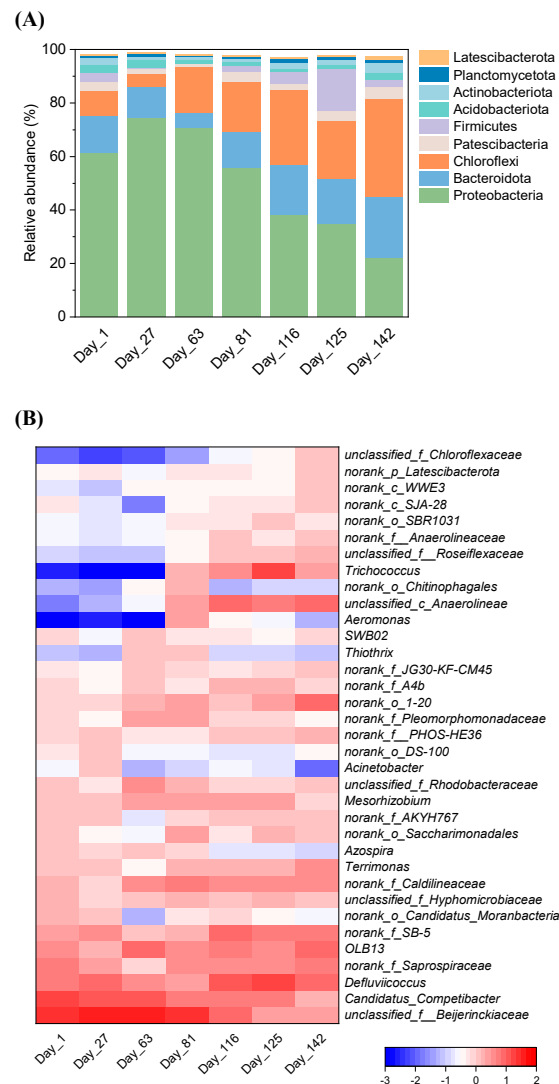


Figure 5. The composition of microbial community (relative abundance > 1% in at least one sample) on the phylum level (A) and genus level (B).

At the genus level (Figure 5B), genera with a relative abundance of >1% in at least one bio-sample were selected and compared in a heatmap. In Day_1, the relative abundances of three genera—*unclassified_f_Bejerinckiaceae* (24.4%), *Candidatus_Competibacter* (16.0%), and *Defluviicoccus* (6.7%)—were > 5%. These three genera remained dominant in Day_27, while *unclassified_f_Bejerinckiaceae* surged to 46.3%. Tamas et al. suggested that some members of Beijerinckiaceae can grow on a wide range of substrates, while others utilize methane and methanol exclusively [24]. Additionally, Qiu et al. reported that the bacterial family Beijerinckiaceae is involved in N cycling [25]. Meanwhile, the relative abundances of other genera were less than 3% in Day_27, except for *norank_f_SB-5* (4.0%). In Day_63, *unclassified_f_Bejerinckiaceae* decreased to 34.2%, while *Candidatus_Competibacter* only decreased slightly to 10.9%. Furthermore, OLB13, *unclassified_f_Rhodobacteraceae*, and *norank_f_Caldilineaceae* slightly increased to 7.6%, 4.1%, and 3.9%, respectively, and maintained a stable relative abundance in the subsequent bio-samples, despite minor fluctuations (Figure 5B). Notably, the relative abundance of *unclassified_f_Bejerinckiaceae* decreased sharply to 3.0% by Day_142, losing its dominant status. However, Xu et al.

reported a significant increase in *unclassified_f_Beijerinckiaceae* (5.9–26.4%) in a similar A/O/A HRT ratio of 2:1:2 in a plug-flow reactor [2], consistent with the phenomenon observed in Day_27. Thus, a plausible explanation is that this genus might be enriched under insufficient aerobic HRT or low-DO conditions, but gradually vanished under sufficient aerobic HRT. Additionally, *Aeromonas* was temporarily increased in Day_81 (3.0%), and *Trichococcus* showed a temporary increase in Day_125 (15.8%). In the final phase (Day_142), five genera—*norank_o_1–20*, *Deftuviicoccus*, *unclassified_c_Anaerolineae*, *OLB13*, and *norank_f_SB-5*—exhibited abundances > 5%.

3.3.3. Functional Groups

AOA systems primarily rely on the coexistence of function bacteria contributing to biological N and P removal, particularly in single-sludge systems [1,2]. Therefore, variations in functional group were analyzed and compared, as shown in Figure 6 and Table S3.

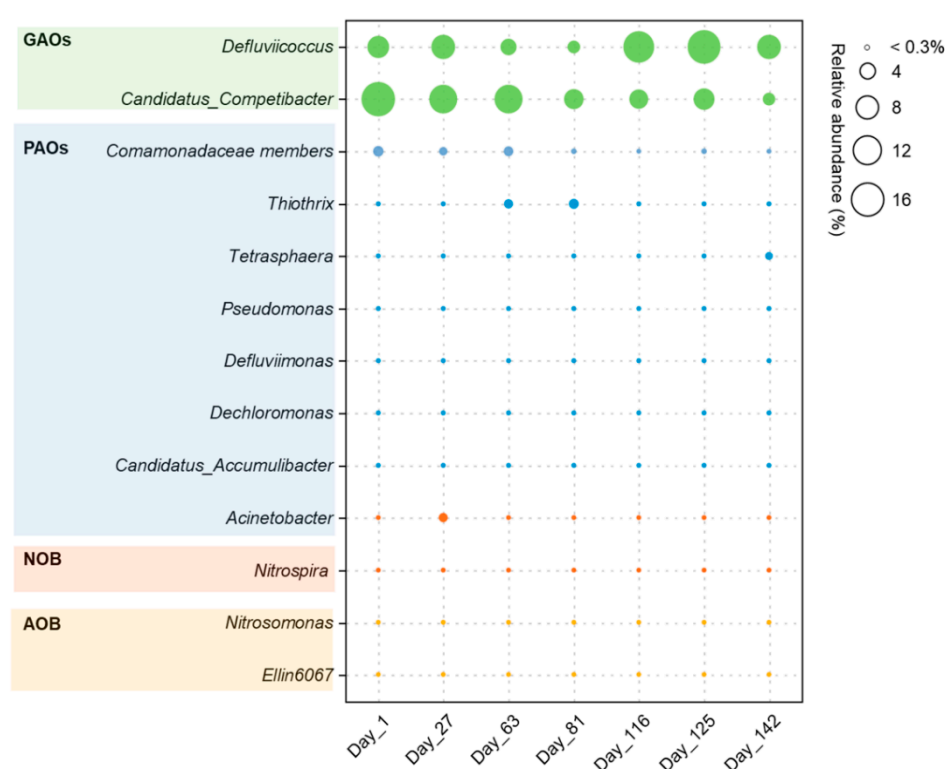


Figure 6. Relative abundance of functional groups in the seven bio-samples.

Regarding nitrifying bacteria, *Ellin6067* and *Nitrosomonas*, commonly classified as AOB [26,27], exhibited comparatively low relative abundances (<0.12%) throughout the operation. Similarly, the typical NOB, *Nitrospira*, exhibited a relative abundance below 0.12%, decreasing further to 0.02% by Day_142 even under a long SRT (100 days). This might be attributed to the inhibition imposed by comparatively short aerobic HRT [2], which prevents NO_2^- -N accumulation in typical cycles (Figure 3). Additionally, in Phase 1, the total relative abundance of nitrifying bacteria decreased from 0.27% to 0.17% (Day_27), consistent with the unsatisfactory NH_4^+ -N removal (Figure 2C). However, nitrifiers were not further enriched despite the operational mode being adjusted to an A/O/A HRT ratio of 1:2:2. Nevertheless, excellent nitrification performance (close to 100%) was maintained, significantly facilitating the TIN removal (Figure 2C,D). This suggests that the nitrifying bacteria play a crucial role in the nitrification, despite their inconspicuous relative abundance; a similar phenomenon was also observed in previous studies [1,2,21].

PAOs, responsible for anaerobic P release and aerobic P uptake, exhibited certain degrees of variation during the operation (Figure 6). In the seed sludge (Day_1), a wide range of PAOs were observed, including *Acinetobacter* (0.30%), *Candidatus_Accumulibacter* (0.01%), *Dechloromonas* (0.02%), *Defluviimonas* (0.12%), *Pseudomonas* (0.06%), *Tetrasphaera* (0.20%), *Thiothrix* (0.10%), and Comamonadaceae members (1.55%) [28]. These PAOs, with total relative abundances of 2.35%, ensured good P removal efficiency (Figure 2B) without requiring a period of domestication. In Phase 2 (A/O/A HRT ratio of 1:2:2, Day_63), sufficient aerobic HRT facilitated the further enrichment of PAOs, increasing their total abundance to 2.88%, primarily due to increases in *Thiothrix* (1.10%) and Comamonadaceae members (1.42%). Moreover, the long SRT might contribute to the growth of *Thiothrix* (1.29% in Day_81), which was also reported to be a type of filamentous bacterium [29]. However, after C/N was adjusted to 4.8 ± 0.3 (Phase 3, Day_81), PAOs exhibited a decreasing trend, dropping to 2.11%, consistent with the deteriorated P efficiency ($34.6 \pm 13.9\%$, Figure 2B). This was primarily attributed to disruption in the balanced competition for carbon sources between PAOs and GAOs [5], resulting in a relative abundance of PAOs below 1.16% in Day_125. Interestingly, when the A/O/A HRT ratio was readjusted to 1:2:2, their abundance recovered to 1.51% (Day_142) and the P removal efficiency returned to its normal level ($96.0 \pm 3.7\%$). Furthermore, denitrifying PAOs (DPAOs), such as *Acinetobacter*, *Candidatus_Accumulibacter*, and *Dechloromonas* [2,30], were observed at certain levels (below 0.30%, except for Day_27), as evidenced by the slight anoxic P uptake in typical cycles (Figure 3).

GAOs, the consistently dominant functional bacteria in the microbial community, mainly included *Candidatus_Competibacter* (ranging from 2.14 to 15.97%) and *Defluviicoccus* (ranging from 2.28 to 15.19%), whereas *Micropruina*, *Nakamurella*, and *Propionivibrio* [2,31] were not observed (Figure 6). Among these, *Candidatus_Competibacter* and *Defluviicoccus* were identified as typical DGAOs [32], which can store intracellular carbon sources (PHAs and Gly) and consume them to perform endogenous denitrification. Due to the presence of DGAOs (22.62%), ED was observed in anoxic compartments in Phase 1 (day 27), contributing to a part of the reduction in NO_3^- -N and NO_2^- -N concentrations (Figure 3A). In Phase 2, the relative abundance of PAOs slightly increased to 2.88% due to the extended aerobic HRT, while GAOs decreased to 14.66%. The competition between PAOs and GAOs weakened the ED and DPR (Figure 2E). On the contrary, the relative abundance of GAOs inclined to 18.30%, while that of PAOs dropped to only 1% in Day_116, corresponding to the improved ED (contributing to approximately 37% TIN removal) and fluctuating P removal performance in Phase 4 (Figures 3C and 2B). This observation supports the opinion of Xu et al., who suggested that PAOs and GAOs share similar ecological niches since both favor alternate anaerobic/aerobic conditions and utilize volatile fatty acids (VFAs) as organic carbon sources [2]. However, Song et al. reported that a GAOs-dominated reactor (relative abundance of 40.98%) was capable of achieving excellent P removal [19]. Additionally, GAOs maintained a high relative abundance (>19%) under insufficient aerobic HRT ratio conditions (Day_1, Day_27, and Day_125), as also reported by Nie et al. (41%) [20] and Xu et al. (23.97%) [2] under low-DO conditions.

3.4. Implications

Setting a reasonable A/O/A HRT ratio is crucial for performing efficient N and P removal while conserving energy in plug-flow AOA systems, particularly under limited HRT conditions. In this study, an optional A/O/A HRT ratio of 1:2:2 was identified; however, an advanced N removal was also achieved at that of 1:1.5:1.5, within a remarkably short HRT of 6 h [14]. The further exploration of more precise and advantageous A/O/A

HRT ratios could involve increasing the number of compartments under suitable conditions, or implementing flexible baffles instead of fixed ones in plug-flow reactors.

To further conserve energy and enhance the ED process, it is important to consider the storage and utilization of intracellular carbon sources, as well as the reduction in aerobic HRT. Reducing aerobic HRT, however, inevitably deteriorates the removal of $\text{NH}_4^+\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ (Figure 2). Liu et al. reported that sludge dual-reflux and mixtures bypassing are able to impose efficient TIN removal (79.9%) at an A/O/A HRT ratio of 2:2:4, even with an aerobic HRT as low as 3.5 h [1]. Moreover, Wang et al. suggested that prolonging anaerobic HRT enhances the storage of intracellular carbon sources, promoting anoxic P uptake and ED [9]. Therefore, it is also essential to optimize the A/O/A HRT ratio when the AOA system is combined with other processes. Additionally, the activity of nitrifying bacteria and the competition between PAOs and GAOs should also be carefully managed, as applying unreasonable A/O/A HRT may lead to the irreparable deterioration of the entire system [2]. Meanwhile, an optimized A/O/A HRT ratio plays an important role in fostering the synergistic interactions among functional bacteria, such as nitrifying bacteria (AOB and NOB), denitrifying bacteria, PAOs, and GAOs, thereby ensuring efficient nutrient removal and energy savings.

4. Conclusions

This study evaluated the performance of a plug-flow AOA reactor under varying A/O/A HRT ratios at a fixed HRT of 13.3 h. Good efficiencies for TIN ($78.3 \pm 5.5\%$), $\text{PO}_4^{3-}\text{-P}$ ($96.0 \pm 3.7\%$), and COD ($79.8 \pm 4.9\%$) were achieved at a reasonable A/O/A HRT ratio of 1:2:2. GAOs, including *Candidatus_Competibacter* (2.1%) and *Defluviicoccus* (8.0%), primarily contributed to a satisfactory ED process, accounting for approximately 37% of TIN removal. The optimized A/O/A HRT ratio promoted synergistic interactions among multiple functional bacteria (AOB, NOB, PAOs, and GAOs), enabling efficient nutrient removal at a low C/N of ~4.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w17050714/s1>, Table S1. The specific composition of the trace elements solutions used in this study. Table S2. The sequencing results, community richness, and diversity estimators of the seven bio-samples. Table S3. Relative abundance of functional bacteria (AOB, NOB, PAOs and GAOs) in the seven bio-samples. The genera with relative abundance < 0.01% are in blue bold and not shown in Figure 6. Table S4. The relative abundance of genera belonging to the family Comamonadaceae.

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Data Availability Statement: The data used in this study are available on request.

Conflicts of Interest: Hao Zheng, Yuange Zheng, Ruitao Gao and Yunjie Jiang were employed by Power China Huadong Engineering Corporation Limited. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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